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Mutagenic Activity of the Photooxidation Products of $C_6H_5NH_2/C_2H_4/NO_x$ Mixtures

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THE MUTAGENIC ACTIVITY OF THE PHOTOOXIDATION PRODUCTS OF $C_6H_5NH_2/C_2H_4/NO_X$ MIXTURES

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ABSTRACT

Mixtures of aniline/ethylene/NO_X were photolyzed in a 22.7-m^3 Teflon reaction chamber operated in a dynamic mode. Several minor products, including nitrobenzene, azobenzene, o-nitroaniline, phenol, and benzoic acid, were identified.

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INTRODUCTION

Aniline ($C_6H_5NH_2$), an aromatic amine, has been investigated as an inhibitor of photochemical smog.[1] As such, it operates by acting as a free radical scavenger to remove hydroxyl radicals (OH) without subsequently producing HO_2. The net result is that NO conversion to NO_2 is slowed, as is the oxidation of available hydrocarbon, and the production of ozone is delayed and inhibited.

Experiments to investigate these effects quantitatively for hydrocarbon and NO_X at near atmospheric concentrations have been performed by Spicer *et al.*[2] The effect of aniline on the oxidation of a hydrocarbon mixture of alkanes, alkenes, and aromatics, was examined at two aniline concentrations. It was confirmed that the NO_2 formation rate was slower with a decreased concentration maximum. In addition, the maximum ozone and peroxyacetyl nitrate (PAN) levels were reduced substantially. However, they did find that a substantial amount of light-scattering aerosol was produced. In these experiments, the light-scattering coefficient, $\Delta\ b_{scat}$, increased nearly two orders of magnitude with the addition of aniline.

It has also been speculated that aniline would be admitted into the atmosphere in significant quantities with the emergence of a synfuel industry. Although this industry has been curtailed, it may become important in the future if natural hydrocarbon energy supplies weaken. The admittance of aniline into the atmosphere, whether intentionally or as a by-product from a production process, raises questions as to the health risks posed both by aniline itself in low-level chronic exposure and from products produced in its atmospheric oxidation. The difficulties in performing toxicological experiments on photooxidation mixtures are twofold. First, the products from the oxidation of aniline have not been well characterized

(thus the kinetics and mechanisms are virtually unknown). Second, the methods for determining adverse health effects of compounds at ambient concentrations are not obvious.

Gas phase exposures of *Salmonella typhimurium* to products from simple hydrocarbon/NO_X photooxidations have recently been performed in an attempt to determine whether mutagenic products are produced and if they are, to identify them.[3] Recent measurements have shown the mutagenicity of products from the ethylene (C_2H_4)/NO_X photooxidation to be nonmutagenic for strains TA98 and TA100 (except for a slight contribution due to formaldehyde [HCHO]). On the other hand, the photooxidation of propylene (C_3H_6)/NO_X mixtures produces highly mutagenic products with TA100 for long extents of reaction.

In the work presented here, $C_6H_5NH_2$/C_2H_4/NO_X mixtures are irradiated in a photochemical reaction chamber. Ethylene, added to provide a radical source for the oxidation of aniline, is used since its photooxidation products are nonmutagenic to the bacterial strains that were used (TA98 and TA100). The mutagenicity assay is then exposed to the effluent from the photooxidation mixture ($C_6H_5NH_2$/C_2H_4/NO_X) to determine the total mutagenic activity as measured with these strains.

EXPERIMENTAL

The experiments were performed in a 22.7-m^3 Teflon photochemical chamber irradiated using a series of blacklights and sunlamps. A schematic of the apparatus has been previously described[4] and is shown in Figure 1. The reactor can be operated in a dynamic mode that requires a steady stream of reactants into the chamber. The effluent is then removed at a rate identical to the total input flow, 50-150 Lpm. The effluent from the chamber is passed through rectangular exposure chambers having a volume of 190 L (with walls coated with fluorocarbon paint). The bioassay test plates reside in these chambers during the exposure.

The reactants, C_2H_4 and NO, were taken directly from diluted (1% in N_2) tank mixtures and metered into the inlet manifold using mass flow controllers. Aniline (Aldrich, 99%) was admitted into the inlet manifold by bubbling N_2 through an impinger bottle containing aniline at 20°C. The reactants were mixed with clean, humidified air before admittance into the chamber.

The sampling and analytical techniques for the measurement of NO, NO_X, O_3,

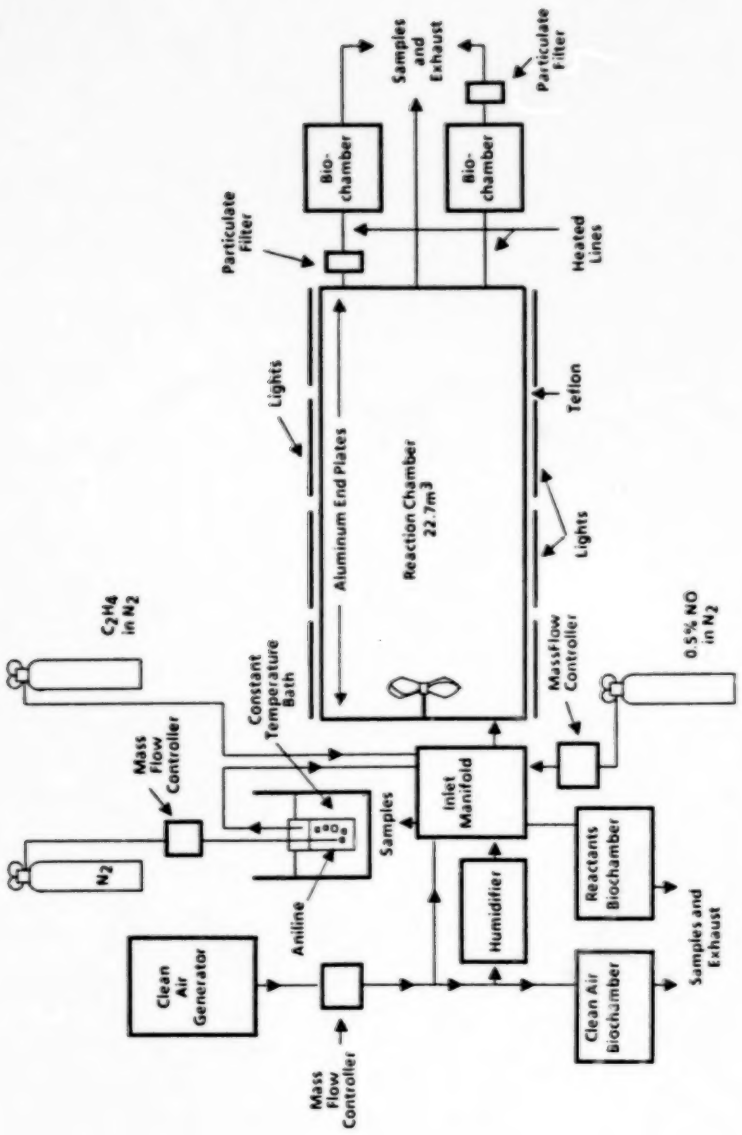

FIG. 1. Experimental Schematic.

HCHO, particle concentration, relative humidity, and physical parameters have already been described.[4] Ethylene was measured using a Varian 1400 gas chromatograph (FID) containing a 3.2-mm x 2-m stainless steel column packed with Carbosieve G. Ethylene standards were prepared by diluting 100 μL of pure C_2H_4 with 100 L pure air in a Teflon bag. Aniline was sampled by bubbling 15 L of the gas sample into glass impinger bottles containing 2 mL of methanol cooled to 0°C. The sampling efficiency for aniline was determined by connecting two bubblers in series and measuring the carry-over in the second bubbler. The efficiency proved to be 100%, within experimental uncertainty. Two-microliter aliquots from this sample were injected into an HP 5840A GC containing a 2-mm x 2-m glass column packed with 10% Silar 10C on 100/120 Gas Chrom P (Allteck Associates, Deerfield, IL). The GC has been modified to use a photoionization detector (PID; HNU Industries) operating with an argon lamp at 10.2 eV. (These detectors are particularly sensitive to most aromatics which generally have ionization potentials in the range of 8-9 eV.) Aniline calibration standards were prepared by diluting the pure compound in methanol. These standards proved to be stable indefinitely.

The biological assay used in this work employs the bacteria, *S. typhimurium*, strain TA98 and TA100. The plates were prepared by adding 0.1 mL of the *S. typhimurium* culture to 2 mL of an agar overlay at 45°C (with or without 0.5 mL of S9 mix). This mixture was then poured onto ~40 mL of plate agar in a glass petri plate. The *S. typhimurium* tester strain TA100 was provided by Dr. Bruce Ames (University of California, Berkeley, CA, USA). Colony counting was done with an Artec 880 automatic colony counter using previously published guidelines.[5] The test procedures used were those of Ames *et al.*,[6] except for the following modifications: (1) glass petri dishes were used, (2) 45 mL of base agar per plate was used, (3) minimal histidine at the same final total concentration was placed in the bottom agar rather than the top agar, and (4) 3 mL of overlay agar with ~1 x 10^8 bacteria were used. The rat-liver homogenate (S9) fraction was prepared from male Charles River CD-1 rats (Wilmington, MO, USA) induced with Aroclor 1254.[6]

In static mode experiments, initial C_2H_4/NO_x and $C_6H_5NH_2/C_2H_4/NO_x$ mixtures were prepared by flowing the reactants into the chamber until they reached the desired concentrations. The reactant gases were then shut off, and dilution air at 14 Lpm allowed for an equivalent sampling rate. The chamber lights were then turned on to start the photooxidation.

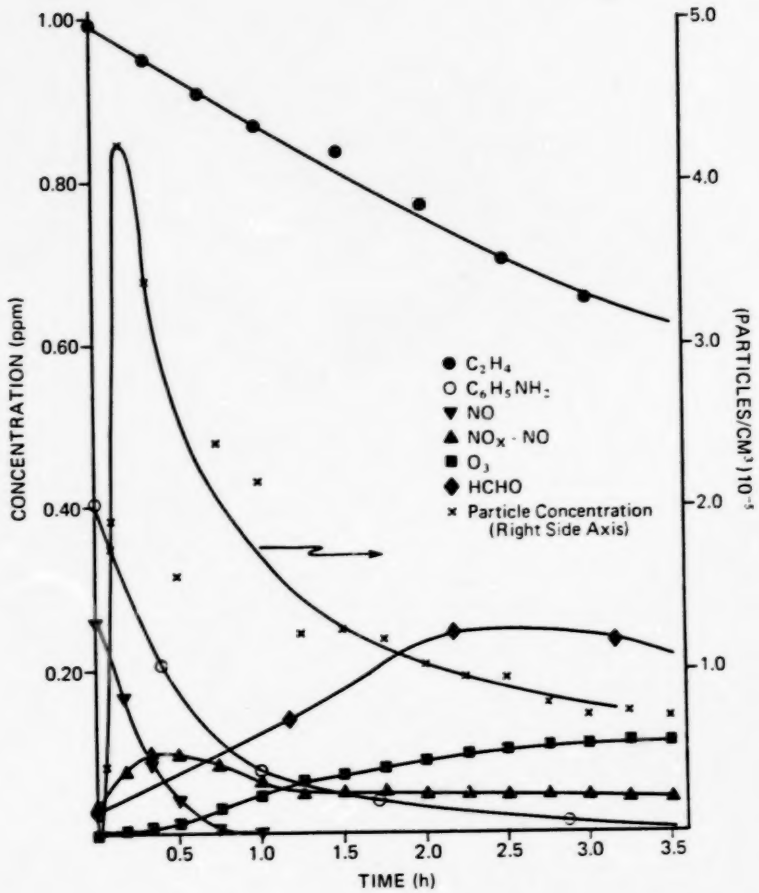

FIG. 2. $C_6H_5NH_2/C_2H_4/NO_X$ Static Irradiation Reactant and Product Profiles.

In the dynamic mode, the experiments were conducted by bringing the irradiated reactant and product concentrations to steady state values in the chamber which is operated at a reactor residence time of $\tau = 2.7$ h, where τ = chamber volume/total flow rate. The effluent was continuously transferred through heated, 9.5-mm Teflon lines to two biochambers (containing the bacteria) at a flow rate of 14 Lpm each. The line to one of the biochambers was filtered to remove possible mutagens present in the aerosol phase. The other biochamber had a filter on the exhaust side to estimate the degree of aerosol deposition in the chamber. In addition, two biochambers were employed as controls to check for mutagenic activity of the clean air and reactant gases.

Two dynamic exposures were performed, one using *S. typhimurium* strain TA98 and the other using TA100. For the exposures, each chamber contained 20 plates with metabolic activation and 20 plates without. Once the biochambers were loaded, they were sealed and the concentrations were allowed to achieve the steady state effluent values. All the plates were then uncovered, starting the exposure. After exposure for 10 h, half of the plates of each type were covered. The gas volume that had passed through each biochamber by this time was 8.5 m^3. The remaining plates continued to be exposed for another 10 h (20 h total). During the exposure, the biochambers were maintained at 25°C. Following the 20-h period, the bacteria were incubated at 37°C for 48 h and then the revertant colonies were counted.

Survivor *S. typhimurium* plates were also placed in the biochamber to monitor possible toxicity of the photochemical mixture. To provide a basis for chemical analysis of the deposited species, surrogate water plates were additionally placed in the chamber. The methods used for measuring compounds deposited into these plates have already been described.[3] Survivor and water plates were covered at both 10 and 20 h.

Particulates were collected on 142-mm diameter glass fiber filters and extracted in methanol using a Soxhlet extractor. The samples were concentrated using a cool blow down procedure. In the final step of the procedure, the extract was solvent exchanged with dimethyl sulfoxide (DMSO). The extracts in DMSO and methanol were used for mutagenicity testing, by using *S. typhimurium* strains TA100 and TA98 (with and without S9) in the plate incorporation procedure previously described.[5]

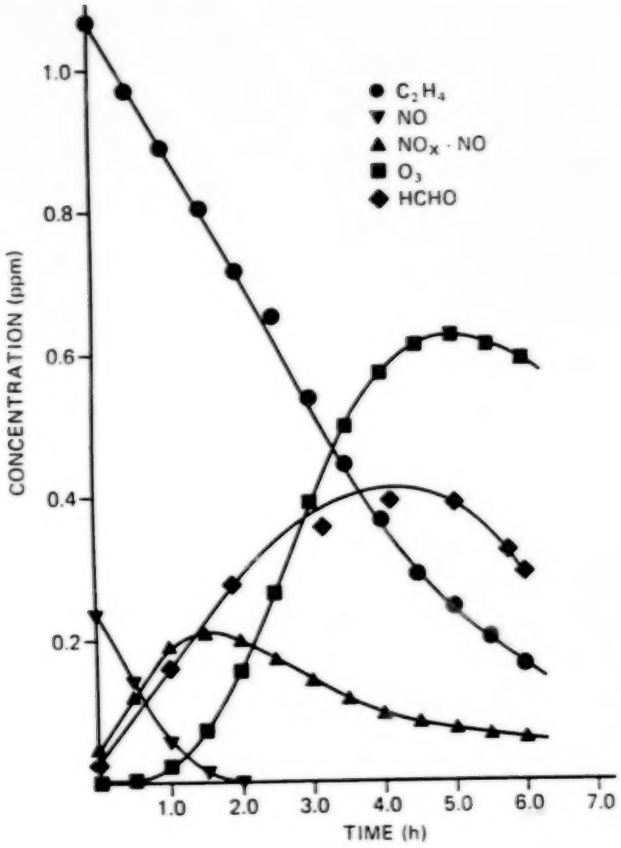

FIG. 3. C_2H_4/NO_X Static Irradiation Reactant and Product Profiles.

TABLE 1. Average Reactant and Product Concentrations (ppm†) for $C_6H_5NH_2/C_2H_4/NO_X$ Exposures at $\tau = 2.7$ h

Species	TA98 Exposure		TA100 Exposure	
	Inlet	Effluent	Inlet	Effluent
$C_6H_5NH_2$	0.252 (0.018)	0.020 (0.003)	0.230 (0.036)	0.014 (0.006)
C_2H_4	1.13 (0.06)	0.714 (0.037)	1.02 (0.03)	0.584 (0.020)
NO	0.231 (0.007)	–	0.188 (0.011)	–
NO_X	0.266 (0.005)	0.063 (0.006)	0.211 (0.008)	0.051 (0.003)
O_3	–	0.178 (0.002)	–	0.203 (0.006)
HCHO	–	0.229 (0.032)	–	0.289 (0.018)
Particles‡	–	1.5×10^3 (0.3×10^3)	–	1.4×10^3 (0.3×10^3)
Relative Humidity	–	37%	–	32%

† standard deviations in parentheses
‡ particle concentration in particles/cm^3

RESULTS

To elucidate the time dependence of the aniline photooxidation, a static mode $C_6H_5NH_2/C_2H_4/NO_X$ irradiation was performed. The initial conditions selected for the static experiment were as follows: $[C_6H_5NH_2]_O$ = 0.40 ppm, $[C_2H_4]_O$ = 1.0 ppm, $[NO]_O$ = 0.25 ppm, and $[NO_2]_O$ = 0.03 ppm. The experiments were conducted at a relative humidity of approximately 35%, and the dilution rate during the experiment was 0.04 h^{-1}. The time profiles for the irradiation having the conditions listed above are given in Figure 2. As shown in the figure, the following compounds were measured as a function of time: C_2H_4, $C_6H_5NH_2$, NO, NO_X, O_3, HCHO, and particulate concentration. A C_2H_4/NO_X photooxidation was performed under essentially the same hydrocarbon/NO_X conditions. ($[C_2H_4]_O$ = 1.07 ppm, $[NO]_O$ = 0.24 ppm, $[NO_2]_O$ = 0.04 ppm.) The time profiles for this photooxidation are given in Figure 3. The results for this experiment are essentially the same as those obtained for previous C_2H_4/NO_X irradiations under these initial conditions.

The $C_6H_5NH_2/C_2H_4/NO_X$ dynamic exposures were conducted under conditions slightly different from the static irradiation. For these experiments, the initial aniline concentration was reduced from that in the static experiment (from 0.40 to 0.25 ppm). The chamber was operated under flow conditions employing an average residence time of 2.7 h. The steady state reactant and product concentrations were

measured periodically during the exposure, and the average concentrations for the two exposures are presented in Table 1.

The mutagenicity test data, including those for the bacteria in the absence and presence of S9, are given in Tables 2 and 3 for TA98 and TA100, respectively. Positive controls (2-nitro-fluorene, 2-aminoanthracene, and sodium azide) were performed appropriately for each experiment. The spontaneous revertants represent laboratory controls under sterile conditions. The survivor plates are used to check for toxicity in the exposure although those shown represent laboratory survivor plates. Chamber survivor plates showed no sign of toxicity in the exposures. The revertant data for the four exposure chambers are presented in the final four rows in each table. Data have been separated for the 10- and 20-h exposures. The clean air biochamber data serve as the baseline data and are the same as the laboratory controls, within experimental uncertainty. The data for the reactants biochamber indicate the degree of mutagenicity of the reactants. The revertant data for the mutagenicity of the photooxidation products are given in the last two rows.

Table 4 is presented to show more clearly the number of revertants in excess of the controls observed for the effluent biochamber test plates. The standard deviations and spontaneous controls used to interpret the data are found in the respective column and row in Tables 2 and 3. In each case, the respective clean air value (i.e., TA98 or TA100, ± S9, 10 or 20 h) is subtracted from the reactants, filtered effluent, and unfiltered effluent data. Although the unfiltered effluent biochamber admits both gas phase and particulate species, the particulate deposition rates may not be sufficient to allow adequate loading of the individual plates. Thus, the particulates collected on the glass fiber filters were solvent extracted and tested for mutagenicity using the plate incorporation test. The data for TA98 are given in Table 5. Filters A and B were located at the entrance of the filtered biochamber during separate experiments. The total mass of each filter was determined gravimetrically. The data have been tabulated with the appropriate spontaneous count (i.e., in methanol or DMSO) subtracted, giving the excess revertant level. The excess revertants vs. dose (μg) has been plotted (Figure 4) to show the degree of linearity present.

TABLE 2. Ames Testing Data (TA98), Revertants/Plate*

	w/o S9		w/ S9	
Spontaneous†	39(3)		52(7)	
3 µg 2-nitrofluorene (-S9)**	268		–	
0.5 µg 2-aminonthracene (+ S9)**	–		536	
Survivors	432		–	
	10 h‡	20 h	10 h	20 h
Clean air biochamber	34(5)	36(6)	55(6)	58(7)
Reactants biochamber	39	38(11)	60(10)	59(8)
Filtered effluent biochamber	47(5)	52(14)	60(7)	54(9)
Unfiltered effluent biochamber	52(8)	50(8)	63(8)	63(10)

* standard deviation in parentheses
† sterile laboratory background controls
** positive control mutagens
‡ exposure time

TABLE 3. Ames Testing Data (TA100), Revertants/Plate*

	w/o S9		w/ S9	
Spontaneous†	186(15)		182(16)	
1 µg sodium azide (-S9)**	438		–	
0.5 µg 2-aminoanthracene (+ S9)**	–		502	
Survivors	298		–	
	10 h‡	20 h	10 h	20 h
Clean air biochamber	169(6)	164(13)	179(15)	184(20)
Reactants biochamber	192(16)	197(11)	206(17)	212(13)
Filtered effluent biochamber	259(22)	291(17)	245(25)	326(37)
Unfiltered effluent biochamber	289(21)	326(35)	279(44)	298(34)

* standard deviation in parentheses
† sterile laboratory background controls
** positive control mutagens
‡ exposure time

TABLE 4. Excess Revertants/Plate†

	TA98 w/o S9		TA98 w/S9	
	10 h	20 h	10 h	20 h
Reactants biochamber	5	2	5	1
Filtered effluent biochamber	18	14	8	5
Unfiltered effluent biochamber	13	16	5	4
	TA100 w/o S9		TA100 w/S9	
	10 h	20 h	10 h	20 h
Reactants biochamber	23	33	27	28
Filtered effluent biochamber	120	162	100	114
Unfiltered effluent biochamber	90	127	66	142

† with respect to number of revertants/plate from the clean air biochamber found in Tables II and III

TABLE 5. Excess Revertants/Plate from the Plate Incorporation of Filter Extracts in TA98*

Filter	Dose (μg)	Revertants/Plate	
		– S9	+ S9
A†	105	390	311
	40	56	49
	4.0	2	26
	0.4	–6	31
B‡	54	198	168
	20	45	70
	2.0	11	38
	0.2	–9	13

* Dose volume: 100 μL.
† Total mass: 4.5 mg.
‡ Total mass: 1.5 mg.

DISCUSSION

The effect of aniline operating as a free radical scavenger can be observed by comparing the results in Figures 2 and 3. The addition of aniline decreased the hydrocarbon oxidation by approximately 40%, at an extent of reaction of 3 h ($\Delta C_2H_4 = 0.61$ ppm without aniline vs. 0.36 ppm with aniline). This effect is observed for an aniline concentration approximately 40% of the initial hydrocarbon. In the dynamic experiments where the aniline concentration has been reduced to approximately 25% of the initial C_2H_4, the inhibition of hydrocarbon oxidation by aniline should be slightly less. (The C_2H_4 decrease observed in the dynamic experiments [Table 1] indicates this to be the case.) In addition to a decrease in C_2H_4 oxidation, the production of HCHO and O_3 is reduced in the presence of aniline. Formaldehyde is decreased from 0.400 to 0.250 ppm at its maximum, while O_3 is decreased by 0.500 ppm. In both cases the concentration maxima occur earlier with the addition of aniline. In addition, the presence of aniline increases the rate of NO_x removal (presumably NO_2) to a greater extent than NO removal. This is consistent with the formation of o-nitroaniline which is observed by GC/MS and formed as follows:

$$C_6H_5NH_2 + OH \rightarrow C_6H_5NH\cdot \xrightarrow{NO_2} C_6H_5NHNO_2$$

Previous studies[1] have indicated that aniline decreases the rate of NO reaction. This is not the case seen by examining Figures 2 and 3. For this experiment

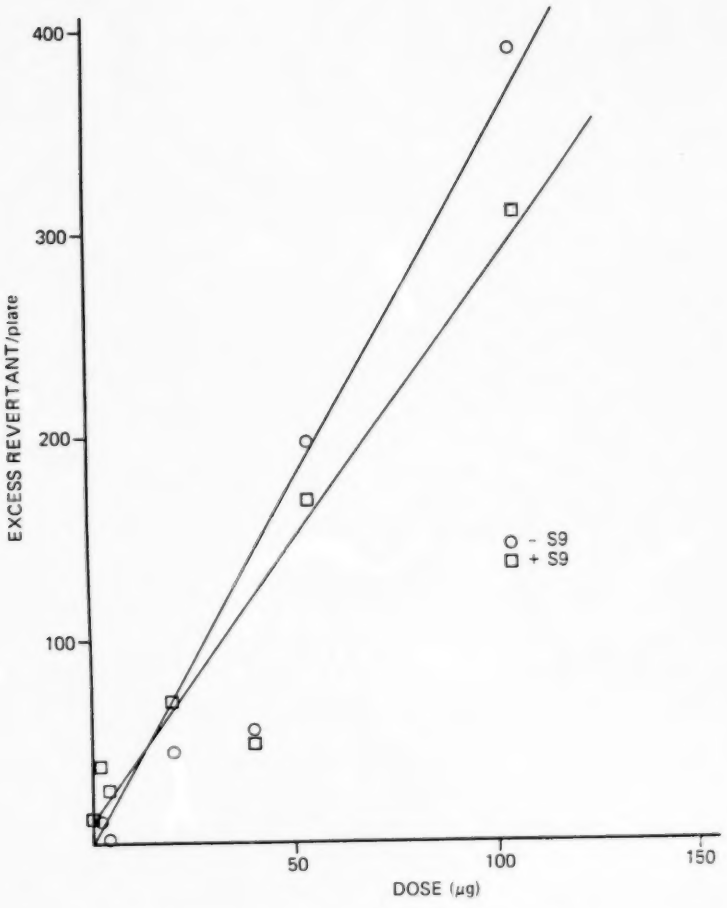

FIG. 4. Mutagenic Activity of Filter Extracts in TA98.

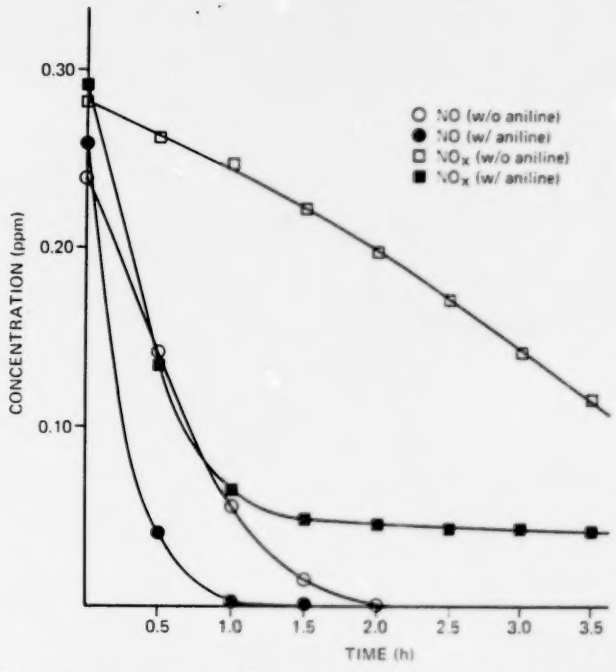

FIG. 5. NO and NO_X Removal Rates in the Presence and Absence of Aniline.

the initial rate of NO removal is a factor of two faster in the presence of aniline. The possibility of a wall source giving a peroxy radical (e.g., HO_2) tends to be discounted by the normal (i.e., reproducible) behavior in the C_2H_4/NO_X photooxidation. An examination of the removal rate of total NO_X (Figure 5) indicates the possible presence of radical intermediates (e.g., $C_6H_5 \cdot NH\cdot$) that remove NO and/or NO_2. Figure 2 shows a rapid increase in particle concentration as soon as aniline begins to be oxidized. The particle concentration decreases with time as the individual particulates begin to coalesce.

Although the initial conditions are slightly different, the steady state concentrations produced in the $C_6H_5NH_2/C_2H_4/NO_X$ dynamic photooxidation (cf.

Table 2) are consistent with that expected from the results of the static experiment at the appropriate extent of reaction, 2.7 h. During the dynamic photooxidation, a search for minor products from the aniline photooxidation was performed. The analysis by GC/MS allowed tentative identification of several minor products including azobenzene ($C_6H_5N=NC_6H_5$), α-nitroaniline ($C_6H_5NHNO_2$), nitrobenzene ($C_6H_5NO_2$), phenol (C_6H_5OH), and benzoic acid (C_6H_5COOH). The gas phase concentrations were estimated with respect to the aniline sensitivity and range from 2-10 ppb. The presence of the latter three products implies the presence of phenyl radicals as intermediates in the photooxidation of aniline.

As seen in Table 4, TA98 under all conditions shows negligible mutagenicity during the exposure. The excess revertants in the filtered and unfiltered chambers ranged from one to two standard deviations higher than the control values. The mutagenicity of the photooxidation products to TA100 is marginal at best. The excess revertants are 3-4 times higher than the standard deviation of the difference, but the absolute value of excess revertants remains less than that of the spontaneous revertants. For the very small response observed there is no distinguishable difference between the filtered and unfiltered chamber and between plates with and without metabolic activation. All data indicate that the reactant mixture is not significantly mutagenic for either TA98 or TA100 at the dose levels of these exposures.

A portion of the minor response observed in TA100 can be accounted for by the weak mutagenicity of HCHO, most of which is formed from the oxidation of C_2H_4. Both standard plate incorporation tests and gas phase exposures with HCHO, as well as other studies,[7] indicate low mutagenic sensitivities in TA100 for HCHO, (approximately 10 revertants/μmole up to 6 μmoles at which point it becomes toxic). The data from the surrogate plates indicate a maximum of 3.2 μmoles going into the plates at 20 h or 32 revertants/plate. The other products have generally not been tested. The fact that they are present in such low concentrations (and standards are not available for some) does not allow us to make an inference regarding their individual mutagenic activities. Whether many of these minor gas phase products will enter the medium is very difficult to establish experimentally, but past experience generally indicates polar oxidation products to be soluble in the medium. The results of the survivor plates in the individual biochambers indicate the

photochemical mixture is in fact not toxic. This is important since toxicity often masks a possible mutagenic response.

The filter extracts show a mutagenic response for TA98 as seen in Figure 4. The mutagenic activity for this response is found to be 3.5 revertants/μg (−S9) and 2.8 revertants/μg (+S9). These values can be used to calculate the expected number of revertants/plate if all particulate matter entered the medium during the exposure. Using the measured particle concentration of 88 $\mu g/m^3$ and 50 plates per exposure chamber, an expected excess revertants level of 53 revertants/plate at 10 h and 105 revertants/plate at 20 h (TA98 without S9) is obtained. This is approximately a factor of five higher than that observed for the unfiltered effluent biochamber (Table 4; TA98 without S9). Thus the conclusion drawn is that the particulates are not being deposited to a significant degree into the medium. For TA100 the filter extracts show no mutagenic activity within the uncertainty of the measurements.

We can, for comparative purposes, examine the results of Kamens *et al.*,[8] in which they measured the mutagenic activity of wood smoke that was oxidized by O_3, NO_2, or a combination of the two. Particulates were collected on filters, solvent extracted, and used in the plate incorporation test. The largest responses that were observed ranged from 1.4 to 2.3 revertants/μg for TA98 without S9. This is about 50% smaller than the mutagenic activity observed for the photooxidation products of aniline. It therefore appears that although the gas phase photooxidation products of aniline are not significantly mutagenic, the particulate matter produced does yield a mutagenic activity that should be considered significant.

CONCLUSIONS

The results of this work show that the gas phase products from the photooxidation of $C_6H_5NH_2/C_2H_4/NO_X$ mixtures show very little if any mutagenic activity in *S. typhimurium* strains TA98 and TA100. Part of the small response in TA100 can be attributed to a contribution from HCHO. The unfiltered exposures indicate that particulates formed do not increase the mutagenic activity. This result is inconsistent with plate incorporation tests with TA98 of the filtered extracts. These results suggest that the particulate deposition during the unfiltered exposure is inefficient. The results of the plate incorporation test indicate that the mutagenicity of particulates from aniline photooxidation is somewhat higher (approximately a factor of two) than that observed in the oxidation of wood smoke by O_3 and NO_2.

DISCLAIMER

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